Flora 209 (2014) 604–612

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Genetic relationship among *Paspalum* species of the subgenus *Anachyris*: Taxonomic and evolutionary implications

Alex L. Zilli[a], Diego H. Hojsgaard[b], Elsa A. Brugnoli[a], Carlos A. Acuña[a], Ana I. Honfi[c], Mario H. Urbani[a], Camilo L. Quarin[a], Eric J. Martínez[a,*]

[a] *Instituto de Botánica del Nordeste, CONICET. Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Corrientes, Argentina*
[b] *Albrecht-von-Haller Institute of Plant Sciences, Department of Systematics, Biodiversity and Evolution of Plants, Georg-August-University of Göttingen, Göttingen, Germany*
[c] *Instituto de Biología Subtropical, CONICET-UNaM. Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Misiones, Argentina*

ARTICLE INFO

Article history:
Received 16 October 2013
Accepted 1 August 2014
Edited by Dr. Rainer Lösch.
Available online 12 August 2014

Keywords:
Anachyris
ISSR markers
Paspalum
Phenotypic variation
Taxonomy

ABSTRACT

Paspalum is one of the most important genera of the Poaceae family due to its large number of species and diversity. The subgenus *Anachyris* comprises six species mainly from South America grouped together by sharing rare spikelet characteristics. A genetic analysis using ISSR markers, compared with the morphological and phenotypic variation observed in each one species, was used to establish genetic relationships among 40 accessions with several ploidy levels, belonging to 5 species of the subgenus *Anachyris*. Fourteen accessions of *Paspalum malacophyllum* (2*x* and 4*x*), 12 of *P. simplex* (2*x*, 3*x*, 4*x* and 6*x*), 4 of *P. procurrens* (2*x* and 4*x*), 4 of *P. usterii* (4*x*) and 6 of *P. volcanensis* (4*x*) were analysed. A total of 227 ISSR loci (98.7% polymorphic) were detected among all accessions, with variable loci number and percentages of polymorphism according to species delimitations. Six main groups were identified by cluster analysis based on Jaccard's genetic distance and UPGMA, four of which matched all the respective accessions of *P. simplex*, *P. procurrens*, *P. usterii* and *P. volcanensis*, while the other two were consistent with two different groups of accessions of *P. malacophyllum*, one involving most tetraploid accessions, and the other one grouping together a tetraploid and two diploid accessions. The distinctive morphological characteristics and the separate clustering of these tetraploid and diploid cytotypes suggest to consider a new multiploid species complex inside the subgenus *Anachyris*. Both cytotypes of *P. procurrens*, and the four co-specific cytotypes of *P. simplex* consistently clustered together forming two specific groups for the two multiploid taxons. This is in agreement with the existence of high phenotypic similarities between diploid and tetraploid cytotypes of *P. procurrens*, and among diploid, triploid, tetraploid and hexaploid cytotypes of *P. simplex*. Since the polyploid cytotypes of these species are reproduced by apomixis, the specific genetic clustering by ISSR markers and morphological and cytological results support the hypothesis that the two multiploid species were originated by autopolyploidy. Our results confirm previous studies suggesting a monophyletic origin for the subgenus *Anachyris* and are concordant with previous data regarding genomic homologies and phylogenetic analyses in the genus.

Introduction

Germplasm banks are current reservoirs of present and recently-past biodiversity of ecosystems on earth. Genetic characterisation of available germplasms is an important step for the conservation, domestication and breeding of plant species. Conservation of plant resources in these germplasm banks comprises different activities, e.g. maintenance, characterisation, or evaluation of genetic diversity within species. The genetic characterisation is carried out to identify accessions and to discern genetic relationship among genotypes (Laurentin, 2009). Plant germplasm is mostly characterised by analysing variation for morphological features and for DNA polymorphisms detected with molecular markers. In contrast to morphological markers, DNA-based techniques have the advantage of being stable, abundant, distributed throughout the genome and independent of environmental influences (Laurentin, 2009; Mondini et al., 2009).

Molecular markers have proved to be valuable tools in the characterisation and evaluation of genetic diversity within and between species and populations. Different molecular markers have been

* Corresponding author. Tel.: +54 379 4427589x146; fax: +54 379 4426218.
E-mail address: eric@agr.unne.edu.ar (E.J. Martínez).

http://dx.doi.org/10.1016/j.flora.2014.08.005
0367-2530/

used to analyse the extent of genetic variation among the diverse groups. For the genus *Paspalum*, among different techniques restriction fragment length polymorphism (RFLP) analysis has been used to establish relationships among 29 species (Jarret et al., 1998). The random amplified polymorphic DNA (RAPD) marker system has been employed also to study the variability in different *Paspalum* species, such as *P. vaginatum* (Liu et al., 1994), *P. scrobiculatum* (M'Ribu and Hilu, 1996), *P. notatum* (Daurelio et al., 2004) and *P. dilatatum* (García et al., 2007). Amplified fragment length polymorphism (AFLP) analysis has been used to characterise the genetic diversity in a germplasm collection of *P. notatum* (Espinoza et al., 2006), while Inter-simple sequence repeat (ISSR) analysis was also used to evaluate the genetic diversity of *P. notatum* in the Brazilian state of Rio Grande do Sul (Cidade et al., 2008) and of another collection of *P. notatum* from Uruguay (Reyno et al., 2012). Microsatellite markers specific for some *Paspalum* species have recently been developed and used in studies on genetic relationships and diversity in *Paspalum* (Cidade et al., 2013). Over the last years, several phylogenetic analyses were performed on different taxonomic groups of *Paspalum*, using morphological characters (Aliscioni, 2002; Denham et al., 2002, 2010; Denham and Zuloaga, 2007; Rua and Aliscioni, 2002), molecular markers (Essi and Souza-Chies, 2007; Giussani et al., 2009; Scataglini et al., 2014; Vaio et al., 2005) and other genetic markers (Cidade et al., 2013; Morrone et al., 2012; Rua et al., 2010; Souza-Chies et al., 2006).

Paspalum is one of the main genera of Poaceae which comprises around 330 species (Zuloaga and Morrone, 2005). According to recent studies on phylogeny, *Paspalum* belongs to a new tribe named Paspaleae, subtribe Paspalineae, which consists of 310 species of American origin (Morrone et al., 2012). In the taxonomic partial revision of the genus, Zuloaga and Morrone (2005) recognised three subgenera: *Anachyris* (Nees) Chase, *Ceresia* (Pers.) Rchb. and the typical subgenus *Paspalum* which includes several unofficial infrageneric categories designed as *Paspalum* groups by Chase (1929). Concurrently, Denham (2005) formally established a fourth subgenus for *Paspalum*, the subgenus *Harpostachys* (Trin.) S. Denham, which includes species transferred from genus *Thrasya* Kunth together with species of the group Decumbentes.

While *Paspalum* is the largest subgenus grouping with about 300 species, *Anachyris* is the less numerous one with only six species (Morrone et al., 2000; Zuloaga and Morrone, 2005). Five of these species are perennial, i.e. *P. malacophyllum* Trin., *P. procurrens* Quarin, *P. simplex* Morong, *P. usterii* Hack. and *P. volcanensis* Zuloaga, Morrone and Denham, while the other one, *P. costellatum* Swallen, is an annual species (Morrone et al., 2000).

The main morphological characteristics of *Anachyris* are related to the spikelets: concavo-convex and boat-shaped, both glumes suppressed (rare with a rudimentary upper glume), and the abaxial surface of the upper lemma conspicuously longitudinally ridged (Chase, 1929; Morrone et al., 2000). Partial phylogenetic analyses of the genus *Paspalum* have shown that the subgenus *Anachyris* is a monophyletic group of six closely-related species (Rua et al., 2010) that share definite morphological, cytological and embryological features (Hojsgaard et al., 2008; Morrone et al., 2000; Urbani et al., 2002).

Interspecific crosses involving *P. simplex, P. procurrens, P. malacophyllum* and *P. usterii* showed the absence of critical hybridisation barriers among these species (Espinoza and Quarin, 1998; Hojsgaard et al., 2008). Meiotic analysis of six different combinations of interspecific hybrids of these 4 species, including homoploid and heteroploid crosses, indicated a common origin and homology among their genomes, and suggested that the whole subgenus *Anachyris* represents an agamic complex unit for which six species are actually recognised (Hojsgaard et al., 2008).

In consequence, it is of worth to know whether species and individuals from the *Anachyris* agamic complex can be identified genetically and differentiated from each other, or if morphologically distinct taxa can be genetically grouped correctly among the different *Anachyris* species. Hence, in the present study we used ISSR markers to analyse the genetic variation occurring in five species of the subgenus with the purpose of (i) identifying genetically cohesive groups among different accessions within and/or among species, and (ii) evaluating whether variable morphologies within the species represent cases of phenotypic plasticity or they segregate genetically from each other indicating the need for a taxonomic reclassification.

Materials and methods

Plant material

The accessions used in this investigation belong to the five perennial *Paspalum* species of the subgenus *Anachyris* (Table 1). *Paspalum costellatum* was not included in this study mainly due to difficulties in obtaining plant material, since the species occurs only in a small area of southern Maranhão State, Brazil, and its annual growth habit critically reduces chances for collection. A total of 40 plants collected in a wide geographical range from southern South America were used for this study. All plants were cultivated in the field and in greenhouses at the Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Corrientes, Argentina (FCA-UNNE). Plants were maintained in field cultivation conditions for the last 5–25 years. The herbarium vouchers representing the living accessions of the analysed material are deposited in several herbaria, mainly in the CTES herbarium (Table 1).

Chromosome counts

The chromosome numbers of 20 living accessions have been determined and communicated in former publications of the research group while original chromosomes counts are established for those accessions that were not previously informed (Table 1). For chromosome counting, root tips were collected from potted plants, placed without fixation in a saturated solution of α-bromonaphthalene for 2 h, and hydrolysed in 1 N HCl for 10 min at 60 °C. Root tips were macerated in a drop of acetocarmine stain, heated, pressed under a cover-slip, and observed using light transmission microscopy.

DNA extraction

Total genomic DNA was isolated by two different methodologies: (1) macro-extraction method using young leaves of adult plant following Martínez et al. (2003), and (2) micro-extraction method using young leaves of seedling plants according to Brugnoli et al. (2013). DNA integrity and concentration were determined by electrophoresis in 1% w/v agarose gels, 1 × TAE buffer (40 mM Tris–HCl, 5 mM NaOAc, 0.77 mM EDTA, pH 8.0) at 40 V for 2 h. DNA dilutions of known concentration were used to estimate the concentration of each DNA sample. DNA samples were visualised under UV light and photographed with GelDoc-It® Imaging System (UVP, LLC), after staining with ethidium bromide (10 mg mL^{-1}). DNA samples were adjusted to 10 ng mL^{-1} for their use in PCR amplifications.

ISSR analysis

A total of 20 ISSR primers (Table 2) were screened for DNA amplification in a preliminary analysis. Finally, 14 primers were selected according to the number of amplified fragments. DNA amplifications were performed according to the methodology described by Cidade et al. (2008) with minor modifications. PCR reactions were performed on 25 μL total volume, containing 10 ng genomic

Table 1
Living plant materials, collection sites, herbarium vouchers, chromosome numbers for each analysed plant and references.

Species	Living accession*	Localities		Herbarium vouchers		Chromosome numbers	
		Country	Latitude–Longitude	Collectors and numbers	Held at**	2*n*	References
P. simplex	C3-32[a]	Argentina	27°16' S–62°18' W	*Quarin* 4109	CTES, US, MO, K	20	Espinoza and Quarin, 1997
	U36[b]	Argentina	26°42' S–60°45' W	*Urbani* 36	CTES, US	30	Urbani et al., 2002
	U45	Argentina	27°19' S–60°41' W	*Urbani* 45	CTES, US, NY	30	Urbani et al., 2002
	Q4168	Argentina	27°27' S–58°48' W	*Quarin* 4168	CTES, US	40	Urbani et al., 2002
	Q3851	Argentina	28°36' S–59°25' W	*Quarin* 3851	CTES, US, F	40	Caponio and Quarin, 1987
	Q4121	Argentina	29°10' S–59°39' W	*Quarin* 4121	CTES, US	40	Urbani et al., 2002
	Q4124	Argentina	28°43' S–59°33' W	*Quarin* 4124	CTES, SI	40	Urbani et al., 2002
	Q4126	Argentina	30°15' S–59°58' W	*Quarin* 4126	CTES	40	This work
	Q4190	Argentina	25°32' S–58°56' W	*Quarin* 4190	CTES, US	40	Urbani et al., 2002
	D&H540	Paraguay	23°31' S–58°46' W	*Daviña & Honfi* 540	MNES, CTES, SI	40	Hojsgaard et al., 2009
	U37[c]	Argentina	29°25' S–57°37' W	*Urbani* 37	CTES, US	60	This work
	U58[c]	Brazil	21°41' S–57°52' W	*Urbani* 58	CTES, K, UTEP	60	This work
P. malacophyllum	V14411	Brazil	27°35' S–50°21' W	*Valls* 14411	CEN	20	Pozzobon et al., 2008
	V14855	Brazil	27°35' S–50°21' W	*Valls* 14855	CEN	20	Pozzobon et al., 2008
	H1191	Argentina	27°28' S–55°47' W	*Honfi* 1191	MNES, CTES	40	This work
	H1257	Argentina	27°30' S–55°27' W	*Honfi* 1257	MNES	40	This work
	DH374	Argentina	25°28' S–64°01' W	*D. Hojsgaard* 374	CTES	40	Hojsgaard et al., 2013
	Q4080	Argentina	24°54' S–63°46' W	*Quarin* 4080	CTES, US, MO	40	This work
	Q4286	Argentina	27°34' S–65°36' W	*Quarin* 4286	CTES, UTEP	40	Hojsgaard et al., 2013
	Q4307	Argentina	32°10' S–64°27' W	*Quarin* 4307	CTES	40	This work
	TK2449	Bolivia	16°07' S–62°01' W	*T. Killeen* 2449	CTES, MO	40	Norrmann et al., 1994
	Q4112	Bolivia	18°29' S–64°06' W	*Quarin* 4112	CTES, US, MO, K	40	This work
	Q4017	Brazil	22°15'S–54°13' W	*Valls* 11813	CEN, CTES	40	Honfi et al., 1990
	V5095	Brazil	18°22' S–49°16' W	*Valls* 5095	CEN, CTES	40	Honfi et al., 1990
	R564	Paraguay	25°15' S–57°15' W	*Rua* 564	BAA, MNES	40	Hojsgaard et al., 2013
	R569	Paraguay	25°11' S–57°19' W	*Rua* 569	BAA, MNES	40	This work
P. procurrens	Q4060	Argentina	25°06' S–64°06' W	*Quarin* 4060	CTES, US, MO, K	20	Quarin, 1993
	DH373	Argentina	25°18' S–64°24' W	*D. Hojsgaard* 373	CTES	40	This work
	DH376	Argentina	25°14' S–64°02' W	*D. Hojsgaard* 376	CTES, CEN	40	This work
	Q4094	Bolivia	20°01' S–63°58' W	*Quarin* 4094	CTES	40	Hojsgaard et al., 2008
P. usterii	H1175	Argentina	27°14' S–55°31' W	*Honfi* 1175	MNES	40	Hojsgaard et al., 2009
	H&D1238	Argentina	27°14' S–55°31' W	*Honfi & Daviña* 1238	MNES	40	This work
	Q3720	Argentina	27°16' S–55°31' W	*Quarin* 3720	CTES	40	This work
	Q4288	Argentina	27°33' S–55°32' W	*Quarin* 4288	CTES	40	This work
P. volcanensis	OM4374	Argentina	23°54' S–65°27' W	*O. Morrone* 4374	CTES, SI	40	This work
	DH370#1[d]	Argentina	23°54' S–65°27' W	*D. Hojsgaard* 370	CTES, CEN	40	This work
	DH370#2[d]	Argentina	23°54' S–65°27' W			40	This work
	DH370#4[d]	Argentina	23°54' S–65°27' W			40	This work
	DH370#5[d]	Argentina	23°54' S–65°27' W			40	This work
	DH370#6[d]	Argentina	23°54' S–65°27' W			40	This work

* Most accessions are single plants obtained from pieces of rhizomes gathered in the site collection together with the collection of the respective herbarium vouchers.
** Herbarium acronyms according to Holmgren and Holmgren (1998).
[a] A plant obtained from seed of the collection Q4109.
[b] A rare triploid plant obtained by seed harvested from the diploid U14 population.
[c] Two hexaploid plants generated by spontaneous 2*n* + *n* fertilisations in two apomictic tetraploid accessions: U37 from Q4168 and U58 from V9073.
[d] Different plants collected in the same population where the herbarium sample D. Hojsgaard 370 was collected.

Table 2
ISSR molecular characterisation of the subgenus *Anachyris*: primers analysed, number of bands amplified, number of polymorphic bands, polymorphism index and polymorphism information content (PIC) obtained by each primer.

Primer repeat 5′ → 3′	Number of bands	Number of polymorphic bands	Polymorphism index (%)	PIC (mean ± SE)
CAG-(AC)7	9	8	88.9	0.28 ± 0.03
(CA)8-T	15	15	100	0.25 ± 0.02
(GT)8-C	18	17	94.4	0.20 ± 0.03
(CT)8-G	13	13	100	0.18 ± 0.02
(AG)8-GC	19	19	100	0.18 ± 0.02
GAG-(AC)7	11	10	90.9	0.21 ± 0.02
(AG)8-C	25	25	100	0.19 ± 0.02
(CTC)6-AC	19	19	100	0.18 ± 0.02
(ATG)5-GA	13	13	100	0.21 ± 0.02
(ACAG)4-GC	17	17	100	0.17 ± 0.02
(GT)8-TC	17	17	100	0.18 ± 0.01
(GA)8-T	14	14	100	0.28 ± 0.01
(AC)8-G	20	20	100	0.14 ± 0.02
(GA)8-C	17	17	100	0.19 ± 0.02
TOTAL	227	224	98.7	

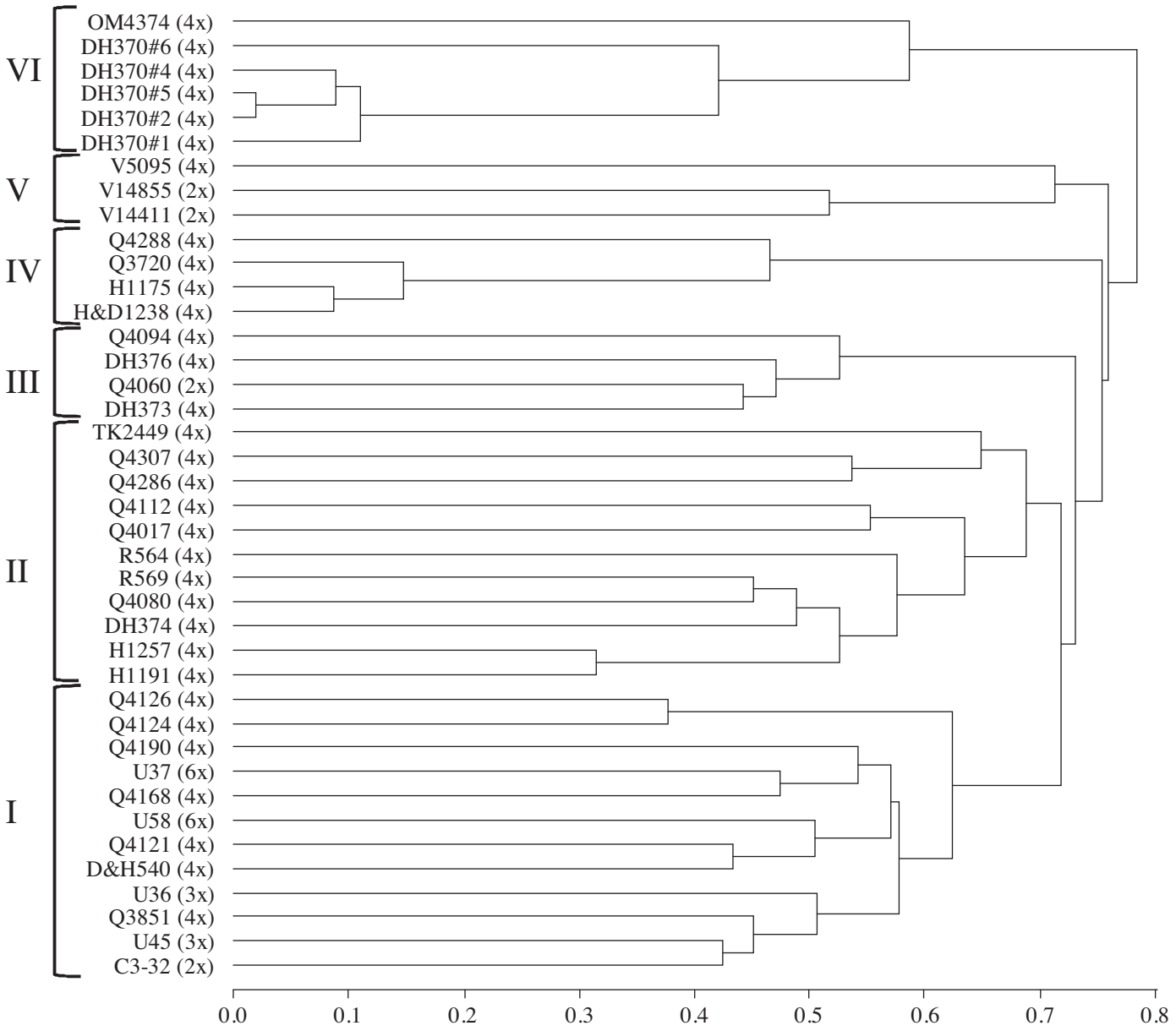

Fig. 1. Dendrogram based on the Jaccard's dissimilarity coefficient and UPGMA among 40 accessions of the subgenus *Anachyris*. The numbers I, II, III, IV, V and VI indicate the six clusters.

DNA, 1× *Taq* polymerase reaction buffer, 100 μM dNTPs, 1 U *Taq* DNA polymerase (Promega) and 0.2 μM of primer. Amplifications were carried out using a Biometra UNO-Thermoblock. Cycles began with 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 45 s at 45–60 °C (depending on the primer) and 2 min at 72 °C, and a final extension of 5 min at 72 °C. DNA samples were amplified twice to verify the reproducibility of the PCR reactions and only consider identical amplification patterns for data analysis. Fifteen microliters of each reaction were electrophoresed in 2% w/v agarose gels/1× TAE (40 mM Tris–HCl, 5 mM NaOAc, 0.77 mM EDTA, pH 8.0) at 60 V for 3 h. Gels were stained with ethidium bromide (10 mg mL^{-1}) and the DNA amplification profiles were visualised and documented with GelDoc-It® Imaging System (UVP).

Data analysis

DNA amplification profiles obtained for each plant were introduced in a binary-data matrix. Only bands that could be unambiguously scored across all the sampled individuals were used in this study. Fragments with the same molecular size were considered as analogous amplicons representing the same locus. The presence of a marker was coded as (1), and absence as (0). The resulting binary-data matrix was analysed using a statistical software package, Info-Gen (Balzarini and Di Rienzo, 2003). Genetic distances among accessions and species were measured by the Jaccard's dissimilarity coefficient (1-S). A dendrogram was constructed based on the matrix of distance using unweighted pair group method with arithmetic averages (UPGMA). A cophenetic correlation coefficient was computed from the clustering matrix in order to assess the goodness-of-fit between the matrix of genetic dissimilarity and the dendrogram.

Results

Chromosome numbers

The chromosome numbers of 20 plants belonging to different accessions were identified in this work (Table 1). Accession Q4168

of *P. simplex* was tetraploid (2*n* = 4*x* = 40) while U37 and U58 were hexaploid (2*n* = 6*x* = 60). All other accessions of *P. malacophyllum*, *P. procurrens*, *P. usterii* and *P. volcanensis*, whose chromosome numbers were not been determined in previous investigations, were tetraploid plants (2*n* = 4*x* = 40).

ISSR polymorphism

The 14 ISSR primers generated a total of 227 identifiable bands (an average of 16 per primer) of which 224 (98.7%) were polymorphic in the 40 plants analysed (Table 2). The number of polymorphic loci evaluated by each primer varied between 8 and 25. The size of the fragments ranged from ~430 to ~2700 bp. Most primers showed a polymorphism index of 100% with a variation range from 88.9 to 100%. Primers CAG-(AC)7 and (GA)8-T showed the highest values of polymorphic information content (PIC) (Table 2).

At the species level, the number of detected loci and the percentage of polymorphic loci were variable. A total of 143 loci were detected among accessions of *P. malacophyllum* of which 135 (94.4%) were polymorphic; while 84 loci were identified for *P. procurrens* of which 60 (71.4%) were polymorphic. Among the accessions of *P. simplex*, 129 loci were detected of which 113 (87.6%) were polymorphic; while in *P. usterii* 63 loci were identified of which 33 (53.2%) were polymorphic. Finally, a total of 72 loci were detected among the accessions of *P. volcanensis* and 50 (69.4%) were found to be polymorphic.

Genetic distance and cluster analysis

The 14 ISSR primers enabled the separation of all the 40 genotypes analysed. The dendrogram generated by using UPGMA resulted in the formation of six major clusters (Fig. 1). A high cophenetic correlation coefficient (0.94) was detected by clustering all plants when using the Jaccard's genetic distance (1-S) based on the average linkage method (UPGMA). Four clusters embraced exactly the accessions corresponding to each one of the following four species: *P. simplex* (cluster I), *P. procurrens* (cluster III), *P. usterii* (cluster IV) and *P. volcanensis* (cluster VI). Eleven accessions of *P. malacophyllum* fell into cluster II, while three other accessions formed cluster V. The Jaccard's dissimilarity coefficient ranged from 0.90 between the most distant (dissimilar) accessions: V14411 (2*x*) of *P. malacophyllum* and Hojs370#1 (4*x*) of *P. volcanensis* to 0.02 between the closest (most similar) accessions: Hojs370#2 (4*x*) and Hojs370#5 (4*x*), which indeed were two plants collected in the same natural population of *P. volcanensis*.

The genetic distances among the species of the subgenus *Anachyris* measured by the Jaccard's dissimilarity coefficient (1-S) ranged from 0.77 to 0.89 (Fig. 2). Clustering of the five species showed a cophenetic correlation coefficient of 0.89, indicating a high goodness-of-fit. The differences in ISSR markers between *P. simplex* and *P. malacophyllum* revealed the lowest genetic distance among the five species, while *P. volcanensis* was the genetically less related species of the group. *Paspalum procurrens* and *P. usterii* were clustered together with a genetic distance of 0.80 between them.

Genetic heterogeneity and phenotypic variability

In order to relate the genetic heterogeneity with morphological and other phenotypic differences among species we summarised in Table 3 the characteristics of the five taxa regarding their biogeography, ploidy levels, growth habit and morphology of their vegetative and generative structures with concern to the six clusters formed, according to molecular variation detected with ISSR markers. Most data related to biogeography and general morphological characteristics of the five species were obtained from the most recent monographic taxonomical treatment of the subgenus

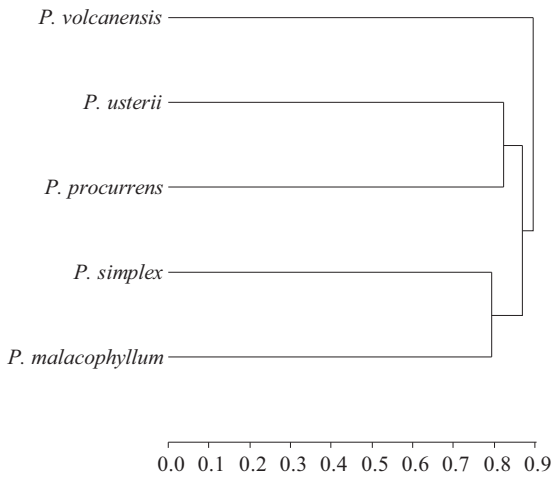

Fig. 2. Clustering based on the Jaccard's dissimilarity coefficient and UPGMA of five *Paspalum* species of the subgenus *Anachyris*.

(Morrone et al., 2000), but also from our own observations on the living plant collection at FCA-UNNE.

There are several specific morphological structures to easily separate a particular species or to group some of them among the subgenus *Anachyris*. For example, the stoloniferous habit identifies *P. procurrens*, the second glume is exclusively present in *P. usterii* and *P. volcanensis*, while the absence of marginal cilias on the rachis of the racemes is characteristic of *P. simplex* and *P. procurrens*. *Paspalum malacophyllum* has the widest range of variation in leaf blade shape and size, even in a single plant.

Only tetraploid forms have been described for *P. volcanensis* and *P. usterii*, while the other three ones are multiploid species which have at least diploid and tetraploid cytotypes, but also triploids and hexaploids in *P. simplex* and hexaploids in *P. malacophyllum*.

Paspalum malacophyllum has a wide distribution in disjunct areas, in Mexico and then in South America, from northeastern Brazil throughout Paraguay, Bolivia and Central Argentina, while *P. usterii* overlaps with *P. malacophyllum* in the southeastern part of its distribution, in southern Brazil, eastern Paraguay, and northeastern Argentina. *Paspalum simplex* is a typical species of the Gran Chaco dry forest, while *P. procurrens* and *P. volcanensis* are distributed in a relatively small area in southern Bolivia and northwestern Argentina. However, these two species inhabit different ecoregions, since *P. procurrens* occurs in the transition area between the Gran Chaco dry forest and near the east border of the Yungas rain forest, while *P. volcanensis* inhabits higher elevations, being found in the Yungas rain forest and the western transition border toward the Prepuna region.

Molecular and taxonomic grouping matched each other almost completely: clusters I, III, IV, and VI, formed according variation detected by ISSR markers, match exactly with the species *P. simplex*, *P. procurrens*, *P. usterii* and *P. volcanensis*, respectively. Eleven tetraploid accessions of *P. malacophyllum* matched cluster II, while the tetraploid accession V5095 and the two diploid ones fell into cluster V.

Discussion

A high percentage of polymorphic ISSR markers were detected among all the accessions of the subgenus *Anachyris*. Even with a relatively low number of individuals in some cases, ISSR markers have been effective in detecting important levels of variation among accessions within a species, allowing us to get a general overview of possible ranges of variability present in natural populations of the *Anachyris* species complex.

Table 3
Comparative analysis among *Paspalum* species of the subgenus *Anachyris*, according to biogeography, ploidy level, morphological phenotypic characteristics and the genetic clustering by ISSR markers. Data on general biological characteristics were compiled from the taxonomic revision of the subgenus (Morrone et al., 2000) and from our own observations.

Characteristic analysed	*P. malacophyllum*	*P. procurrens*	*P. simplex*	*P. usterii*	*P. volcanensis*
Biogeography	Disjunct distribution: Mexico in the North and Brazil, Bolivia, Paraguay and Argentina in South America. Rocky hillsides, roadsides or forest edges, from sea level to 3000 m altitude	Northwestern Argentina and southern Bolivia. Semiarid phytogeographic Chaco region, on sandy and rocky soils, from 350 to 800 m altitude	Chaco phytogeographic region: eastern Bolivia, western Paraguay, and northern Argentina; then southward to extreme southern Brazil and northern Uruguay; open, dry environments, from sea level to 500 m altitude	Central-eastern Brazil to eastern Paraguay and Misiones province in Argentina. Forest edges, on sandy-clay soils, from 200 to 1300 m altitude	A narrow distribution area between the Yungas forest and the Puna region, both sides of the Argentina-Bolivia border, on rocky and sandy soils, from 1000 to 3000 m altitude
Ploidy levels	2*x*, 4*x*, 6*x*	2*x*, 4*x*	2*x*, 3*x*, 4*x*, 6x	4*x*	4*x*
Growth habit	Tufted culms from short scaly rhizomes	Stoloniferous	Tufted culms from short scaly rhizomes	Robust clumps from long arched, stout, scaly rhizomes	Tufted culms from short arched rhizomes
Plant height (m)	0.70–2.0	0.45–0.85	0.30–0.90 (−1.20)	1.0–2.0	0.45–0.60
Leaf blade shape	Elongate, linear-lanceolate to lanceolate. The lower and middle ones tapering to a narrow base, pseudo-peciolate	Lanceolate, rounded at base	Linear-acuminate	Linear-acuminate, the lower ones tapering to the base	Linear-lanceolate, rounded at base and acuminate apex
Leaf blade length (cm)*	13–40	5–20	10–25	15–30	12–22
Leaf blade width (cm)*	0.8–2.5(−4)	0.5–2.0	0.2–0.6	0.8–2.5	0.7–1.3
Spikelets with second glume present and pubescent	–	–	–	+	+
Rachis of the racemes long-ciliate on the margins	+	–	–	+	+
Clusters**	II and V	III	I	IV	VI

* Measurements implicate the second leaf below the inflorescence.
** Data obtained from the variation detected by ISSR markers based on the Jaccard's genetic distance and UPGMA.

Clustering measured by the Jaccard's dissimilarity coefficient (1-S) and UPGMA analyses detected six main groups, four of which are actually coincident with four botanically recognised taxa that were delimitated with regard to their morphological characteristics. The two remaining clusters contained all those accessions whose herbarium vouchers have been classified as *P. malacophyllum*.

All accessions of *P. simplex*, including diploid, triploid, tetraploid and hexaploid cytotypes, formed a single cluster. The accessions evaluated in *P. simplex* correspond to representative sampling all over the natural distribution of the species. Most of the distribution area of *P. simplex* is occupied by apomictic tetraploid individuals. Diploid populations are restricted to a relatively small

Fig. 3. Rhizomatous basal section of *P. malacophyllum*. **a** Short scaly rhizomes with tufted culms representative of the species (accession H1191). **b** Pachymorph rhizomes forming clumps of loosely emerging culms in the particular accession V5095. Scale bar = 5 cm.

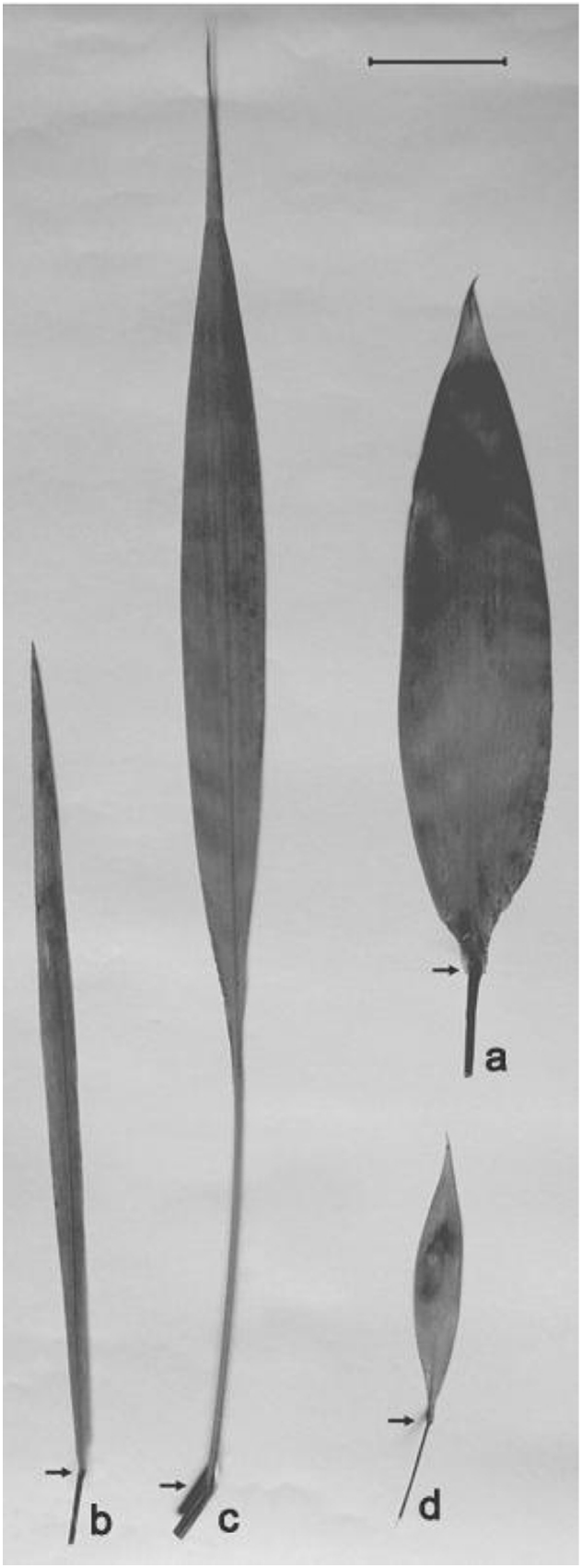

Fig. 4. Different types of leaf blades from middle flowering culms observed in *P. malacophyllum*. **a** Atypical shape and size of a leaf blade of accession V5095, exceptionally wide, lanceolate and shortly tapering to a rounded base. **b** Slender, elongated and non-petiolate leaf blade of accession H1191. **c** Leaf blade of accession TK2449, with linear-lanceolate shape and largely pseudo-peciolate. **d** Leaf blade of the diploid accession V14855 closely resembling the shape of tetraploid accession V5095 but with a much smaller size. Arrows indicate the connection of the leaf blade with the leaf-sheath. Scale bar = 5 cm.

part of the whole area of distribution growing in sympatry or being allopatric with the tetraploid cytotype. Hexaploid plants were always integrated with tetraploid populations, while only two triploid plants were found so far, one in a diploid and the other in a mixed diploid–tetraploid population. Morphologically, all cytotypes are very similar and there are no particular phenotypes to identify ploidy levels (Urbani et al., 2002). The genetic similarities observed among diploids, triploids and tetraploids, as well as between tetraploids and hexaploids suggest a strong genetic relatedness among them and support the hypothesis that all polyploid cytotypes of *P. simplex* originated by autoploidy (Hojsgaard et al., 2008; Urbani et al., 2002). Except for one rare sexual triploid plant, all other polyploids reproduce by apomixis. Thus, *P. simplex* constitutes an agamic complex with different polyploid races which originated by autoploidy from the co-specific sexual diploid cytotype. In *Paspalum*, diploids are predominantly sexual and usually allogamous. A two-step mechanism of polyploidisation and gene flow among ploidy levels has been described (Quarin et al., 1989) through the formation of triploid individuals mobilising complete genomes to higher ploidy levels, as expected from the accepted two-step sexual polyploidisation mechanism (Stebbins, 1971). Being *P. simplex* a multiploid complex, triploids must act as a bridge for gene transfer between diploids and tetraploids, as they were found in sympatric diploid–tetraploid populations (Brugnoli et al., 2013; Urbani et al., 2002). If this is the case, observed levels of diversity among *P. simplex* genotypes, even asexual reproducing polyploids which should show lower degrees of polymorphisms, can be explained as a consequence of temporary triploids individuals injecting variability in all ploidy levels, maintaining a roughly unique morphotype.

A higher genetic affinity between *P. simplex* and *P. malacophyllum* was observed which is consistent with the taxonomic treatment given to the subgenus *Anachyris* by Morrone et al. (2000). *Paspalum simplex* differs from *P. malacophyllum* by having smaller sized plants with shorter and narrower leaf blades, relaxed inflorescences and with fewer racemes (Morrone et al., 2000).

Most tetraploid accessions of *P. malacophyllum* were clustered in a second group depicting a high variation in genetic distance among them. *Paspalum malacophyllum* is the most widely distributed species and shows the greatest morphological variation among *Anachyris* species (Morrone et al., 2000). Four taxons previously considered as close relatives of *P. malacophyllum* have been included in the synonymy of this species after Morrone et al. (2000) carried out a phenetic analysis, using thirty morphological characters, without finding enough evidence to maintain separation. On the other hand, the tetraploid accession V5095 is a special case. This accession was grouped away from the other 11 tetraploid accessions of *P. malacophyllum* considered in our analysis, but close to the diploid V14411 and V14855 accessions. The accession V5095 can be easily distinguished from other tetraploid accessions of the species mainly by the morphology of the rhizomes and its shorter, wider, and distinctly-shaped leaves. Typically, *P. malacophyllum* has short stout scaly rhizomes from which emerge tufted culms forming caespitose plants (Fig. 3a). The accession V5095 has pachymorph rhizomes forming clumps of loosely emerging culms, multinodes, somewhat decumbent with long ascending or upright ends (Fig. 3b). The leaf blades of V5095 (Fig. 4a) are exceptionally wide for the species, from 4 to 7.5 cm width and usually 20–30 cm long, lanceolate with rounded or cordate base in the uppermost leaves of the culms, to lanceolate and shortly tapering to a narrow base, though never pseudo-petiolate, in the middle or lower part of the culms. In a different way, the typical tetraploid

accessions of *P. malacophyllum* have elongate to linear-lanceolate leaf blades (Fig. 4b), and usually the leaves of the middle and lower part of the culms have lanceolate blades largely tapering to the base, forming a pseudo-petiole (Fig. 4c). In spite of the differences in the leaf-blades shape, the relation between width and length of leaf blades is constantly different between accessions of cluster II and the three accessions of cluster V. For example, we observed that leaf blades of the most distant accessions among the cluster II (see dendrogram, Fig. 1) are approximately 17 times longer than wide in H1191 and 25 times in TK2449, while in V5095 is merely 4–5 times longer than wide and just about 6 times in the diploids V14411 and V14855. All tetraploid accessions of cluster II have a width:length leaf-blades ratio that varies between 0.04 and 0.08, while this ratio is much higher in the two diploid accessions and in tetraploid V5095 (mean range 0.16–0.26). Interestingly, the growth habit, the rhizome system, and the leaves of the two diploid accessions, V14411 and V14855, closely resemble the tetraploid V5095, though the diploids have very slender rhizomes and the leaf blades have generally a much smaller size than V5095 (Fig. 4d) and, in addition, the middle culm leaves have shortly pseudo-petiolate blades. Unfortunately, the three accessions, tetraploid V5095 and diploid V14411 and V14855, which clustered separately in our genetic analysis, were not included in the original phenetic analysis of *P. malacophyllum* surveyed in the taxonomic monography of subgenus *Anachyris* conducted by Morrone et al. (2000). Notwithstanding, it was pointed out in this monography that *P. malacophyllum* has a great variation in the width of leaf-blades and some herbarium collections with wide blades were mentioned. We have the opportunity to see the photographs of two of them, deposited in the herbarium of Missouri Botanical Garden: collections Irwin et al. # 15249 and # 27288. The former is a robust plant quite similar in leaf blades shape and size to our accession TK2449, while # 27288 from Brazil, Minas Gerais, Serra do Cabral, ca. 2.5 km W of Cantoni, looks very similar in leaf blade shape and size to our accession V5095 collected by Valls in Brazil, Goias, Itumbiara way to Bom Jesus de Goias. The accession V5095 has already been considered an atypical plant for *P. malacophyllum*. Moreover, it was suggested that it might represent a different taxon together with diploid V14411 and V14855 accessions due to their morphological characteristics and their decumbent growth habit (Hojsgaard et al., 2008). Our results suggest that the accessions of cluster V would merit a specific taxonomic category in which the accessions V14411 and V14855 belong to the diploid and accession V5095 to the tetraploid cytotype of a new multiploid *Paspalum* species of the subgenus *Anachyris*. Anyway, molecular data indicate that *P. malacophyllum* is a highly variable species in which it is possible to distinguish, in South American representative collections, at least two main groups according to the variation detected by ISSR markers and morphological characteristics. Indeed, the species has a disjunct distribution with a northern area in Mexico and a most important one in South America, from the Equator circle southward to central Argentina (Morrone et al., 2000). Unfortunately we missed the chance to analyse collections of *P. malacophyllum* from the North Hemisphere in order to cover the entire distribution area of the species.

The four accessions of *P. procurrens* formed a well-defined cluster including one diploid and three tetraploid accessions. The clustering indicated a closer genetic relation with *P. simplex* and most *P. malacophyllum* representatives than with other species of *Anachyris* in coincidence with the observation of Morrone et al. (2000) that *P. procurrens* is a species closely related to *P. malacophyllum* and *P. simplex* differing from both by its stoloniferous growth habit. Moreover, the results from crosses between *P. simplex* and *P. procurrens* and from the cytogenetic analysis of their interspecific hybrids (Hojsgaard et al., 2008) have indicated that both species share the same basic genome, and that the stoloniferous growth habit is a dominant trait. However, our analysis with ISSR markers suggests that there are other important genetics differences that validate *P. procurrens* as a well-defined species in the subgenus *Anachyris*.

All accessions of *P. usterii* clustered together and fell separate from other species of *Anachyris* in our analysis with ISSR markers. Morrone et al. (2000) have suggested that *P. usterii* has a higher affinity with *P. volcanenesis* than with other species of the subgenus because these two species have pubescent spikelets which bear an upper glume, the bract that is absent in all other species of *Anachyris*. However, these two species are geographically separated since *P. usterii* is distributed in northeast Argentina, southern Brazil, and eastern Paraguay, while *P. volacanensis* grows in northwestern Argentina and southern Bolivia. Our analysis indicates an important genetic distance between *P. usterii* and *P. volcanensis*. As was expected, there was no great genetic variation among *P. usterii* accessions, since the species reproduces by facultative apomixis (Hojsgaard et al., 2008) and the analysed accessions were collected in a relatively small area in the Province of Misiones, Argentina. The narrow genetic distance observed among the accessions of *P. usterii* H1175, Q3720 and H & D1238 is because of its apomictic reproduction mode, and they were collected in very close sites to each other. This could be explained by a very recent common genetic origin of the three accessions.

The accessions of *P. volcanensis* clustered as the most genetically distant group among all here investigated accessions of *Anachyris*. Despite the fact that all of them are indeed different plants of one and the same population, an important degree of genetic variation was observed among them. The species is still poorly investigated with respect to its general biological characteristics, mainly concerning its reproductive system and polyploid origin. Further research on these subjects would help understanding the biology and the evolutionary relations of this species.

Genetic analysis carried out with ISSR markers on an important number of accessions belonging to five of the recognised six *Paspalum* species of the subgenus *Anachyris* permitted us to corroborate the current taxonomic delimitations of these species. The results suggest that some collections until now considered as part of *P. malacophyllum* belong to a new taxonomic entity, but to be sure about this, further studies, also at the phenotypic level, are needed. The methodology used in this work could be useful for the interpretation of the morphological variation observed in other taxonomic group of *Paspalum* or even in other genera of Paniceae.

Acknowledgements

We thank Professor Michael D. Hayward, Aberystwyth, Wales, UK, for his critical review and suggestions related to English grammar. This study was financed by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP-11220080101378), the Agencia Nacional de Promoción Científica y Técnica (ANPCYT, PICT-2008-00264), and the Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste (UNNE, PI-046-06), Argentina. A.L. Zilli and E.A. Brugnoli received a fellowship from CONICET and ANPCYT-UNNE, respectively. D.H. Hojsgaard is Lecturer and researcher of the University of Goettingen, Germany. A.I. Honfi is Professor of the Universidad Nacional de Misiones (UNaM) and researcher of the Instituto de Biología Subtropical (CONICET-UNaM). M.H. Urbani is Professor and researcher of the UNNE. C.A. Acuña, E.J. Martínez, and C.L. Quarin are Professors of the UNNE and members of the research career of CONICET.

References

Aliscioni, S.S., 2002. Contribución a la filogenia del género *Paspalum* (Poaceae: Panicoideae: Paniceae). Ann. Mo. Bot. Gard. 89, 504–523.

Balzarini, M., Di Rienzo, J., 2003. Info-Gen: Software para análisis estadístico de datos genéticos. Facultad de Ciencia Agropecuarias. Universidad Nacional de Córdoba, Argentina.
Brugnoli, A.E., Urbani, M.H., Quarin, C.L., Martínez, E.J., Acuña, C.A., 2013. Diversity in diploid, tetraploid and mixed diploid–tetraploid populations of *Paspalum simplex*. Crop Sci. 53, 1509–1516.
Caponio, I., Quarin, C.L., 1987. El sistema genético de *Paspalum simplex* y de un híbrido interespecífico con *P. dilatatum*. Kurtziana 19, 35–45.
Chase, A., 1929. The South American Species of *Paspalum*. Unpublished manuscript.
Cidade, F.W., Dall'Agnol, M., Bered, F., De Souza-Chies, T.T., 2008. Genetic diversity of the complex *Paspalum notatum* Flügge (Paniceae: Panicoideae). Genet. Resour. Crop Evol. 55, 235–246.
Cidade, F.W., et al., (8 authors), 2013. Genetic variation in polyploid forage grass: assessing the molecular genetic variability in the *Paspalum* genus. BMC Genet. 14, 50.
Daurelio, L.D., Espinoza, F., Quarin, C.L., Pessino, S.C., 2004. Genetic diversity in sexual diploid and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry or allopatry. Plant Syst. Evol. 244, 189–199.
Denham, S.S., 2005. Revisión sistemática del subgénero *Harpostachys* de *Paspalum* (Poaceae: Panicoideae: Paniceae). Ann. Mo. Bot. Gard. 92, 463–532.
Denham, S.S., Zuloaga, F.O., 2007. Phylogenetic relationships of the Decumbentes group of *Paspalum*, *Thrasya*, and *Thrasyopsis* (Poaceae: Panicoideae: Paniceae). Aliso 23, 545–562.
Denham, S.S., Zuloaga, F.O., Morrone, O., 2002. Systematic revision and phylogeny of *Paspalum* subgenus Ceresia (Poaceae: Panicoideae: Paniceae). Ann. Mo. Bot. Gard. 89, 227–399.
Denham, S.S., Morrone, O., Zuloaga, F.O., 2010. Estudios en el género *Paspalum* (Poaceae, Panicoideae, Paniceae). *Paspalum denticulatum* y especies afines. Ann. Mo. Bot. Gard. 97, 11–33.
Espinoza, F., Quarin, C.L., 1997. Cytoembryology of *Paspalum chaseanum* and sexual diploid biotypes of two apomictic *Paspalum* species. Aust. J. Bot. 45, 871–877.
Espinoza, F., Quarin, C.L., 1998. Relación genómica entre citotipos diploides de *Paspalum simplex* y *P. procurrens* (Poaceae, Paniceae). Darwiniana 36, 59–63.
Espinoza, F., Daurelio, L.D., Pessino, S.C., Valle, E.M., Quarin, C.L., 2006. Genetic characterisation of *Paspalum notatum* accessions by AFLP markers. Plant Syst. Evol. 258, 147–159.
Essi, L., Souza-Chies, T.T., 2007. Phylogeny of Linearia and Notata groups of *Paspalum* L. (Poaceae, Panicoideae, Paniceae) and related species. Genet. Resour. Crop. Evol. 54, 779–791.
García, M.V., Balatti, P.A., Arturi, M.J., 2007. Genetic variability in natural populations of *Paspalum dilatatum* Poir. analysed by means of morphological traits and molecular markers. Genet. Resour. Crop Evol. 54, 935–946.
Giussani, L.M., Zuloaga, F.O., Quarin, C.L., Cota-Sánchez, H., Ubayasena, K., Morrone, O., 2009. Phylogenetic relationships in the genus *Paspalum* (Poaceae: Panicoideae: Paniceae): an assessment of the Quadrifaria and Virgata informal groups. Syst. Bot. 34, 32–43.
Hojsgaard, D.H., Schegg, E., Valls, J.F.M., Martínez, E.J., Quarin, C.L., 2008. Sexuality, apomixis, ploidy levels, and genomic relationships among four *Paspalum* species of the subgenus *Anachyris* (Poaceae). Flora 203, 535–547.
Hojsgaard, D.H., Honfi, A.I., Rua, G.H., Daviña, J.R., 2009. Chromosome numbers and ploidy levels of *Paspalum* species from subtropical South America (Poaceae). Genet. Resour. Crop Evol. 56, 533–545.
Hojsgaard, D.H., Martínez, E.J., Quarin, C.L., 2013. Competition between meiotic and apomictic pathways during ovule and seed development results in clonality. New Phytol. 197, 336–347.
Holmgren, P.K., Holmgren, N.H., 1998. Index Herbariorum: A Global Directory of Public Herbaria and Associated Staff. New York Botanical Garden's Virtual Herbarium (continuously updated) http://sweetgum.nybg.org/ih/
Honfi, A.I., Quarin, C.L., Valls, J.F.M., 1990. Estudios cariológicos en gramíneas sudamericanas. Darwiniana 30, 87–94.
Jarret, R.L., Liu, Z.W., Webster, R.W., 1998. Genetic diversity among *Paspalum* spp. as determined by RFLPs. Euphytica 104, 119–125.
Laurentin, H., 2009. Data analysis for molecular characterisation of plant genetic resources. Genet. Resour. Crop Evol. 56, 277–292.
Liu, Z.W., Jarret, R.L., Duncan, R.R., Kresovich, S., 1994. Genetic relationships and variation among ecotypes of seashore paspalum (*Paspalum vaginatum*) determined by random amplified polymorphic DNA markers. Genome 37, 1011–1017.
Martínez, E.J., Hopp, H.E., Stein, J., Ortiz, J.P.A., Quarin, C.L., 2003. Genetic characterisation of apospory in tetraploid *Paspalum notatum* based on the identification of linked molecular markers. Mol. Breed 12, 312–327.
Mondini, L., Noorani, A., Pagnotta, M.A., 2009. Assessing plant genetic diversity by molecular tools. Diversity 1, 19–35.
M'Ribu, H.K., Hilu, K.W., 1996. Application of random amplified polymorphic DNA to study genetic diversity in *Paspalum scrobiculatum* L. (*Kodo millet*, Poaceae). Genet. Resour. Crop Evol. 43, 203–210.
Morrone, O., Denham, S.S., Aliscioni, S.S., Zuloaga, F., 2000. Revisión de las especies de *Paspalum* (Panicoideae: Paniceae), subgénero *Anachyris*. Candollea 55, 105–135.
Morrone, O., et al., (10 authors), 2012. Phylogeny of the Paniceae (Poaceae: Panicoideae): integrating plastid DNA sequences and morphology into a new classification. Cladistics 28, 333–356.
Norrmann, G.A., Quarin, C.L., Killeen, T., 1994. Chromosome numbers in Bolivian grasses. Ann. Mo. Bot. Gard. 81, 768–774.
Pozzobon, M.T., et al., (8 authors), 2008. Cytogenetic analyses in *Paspalum* L. reveal new diploid species and accessions. Cienc. Rural 38, 1292–1299.
Quarin, C.L., 1993. Morfología, citología y sistema reproductivo de una nueva especie de gramínea: *Paspalum procurrens*. Bol. Soc. Argent. Bot. 29, 73–76.
Quarin, C.L., Norrmann, G.A., Urbani, M.U., 1989. Polyploidization in aposporous *Paspalum* species. Apomixis Newsletter 1, 28–29.
Reyno, R., et al., (9 authors), 2012. Molecular and cytogenetic characterisation of a collection of bahiagrass (*Paspalum notatum* Flügge) native to Uruguay. Genet. Resour. Crop Evol. 59, 1823–1832.
Rua, G.H., Aliscioni, S.S., 2002. A morphology-based cladistic analysis of *Paspalum* sect *Pectinata*. Syst. Bot. 27, 489–501.
Rua, G.H., Speranza, P.R., Vaio, M., Arakaki, M., 2010. A phylogenetic analysis of the genus *Paspalum* (Poaceae) based on cpDNA and morphology. Plant Syst. Evol. 288, 227–243.
Scataglini, M.A., Zuloaga, F.O., Giussani, L.M., Denham, S.S., Morrone, O., 2014. Phylogeny of New World *Paspalum* (Poaceae, Panicoideae, Paspaleae) based on plastid and nuclear markers. Plant Syst. Evol. 300, 1051–1070.
Souza-Chies, T.T., Essi, L., Rua, G.H., Valls, J.F.M., Miz, R., 2006. A preliminary approach to the phylogeny of the genus *Paspalum* (Poaceae). Genetica 126, 15–32.
Stebbins, G.L., 1971. Chromosomal Evolution in Higher Plants. Addison-Wesley, London.
Urbani, M.H., Quarin, C.L., Espinoza, F., Penteado, M.I.O., Rodrigues, I.F., 2002. Cytogeography and reproduction of the *Paspalum simplex* polyploid complex. Plant Syst. Evol. 236, 99–105.
Vaio, M., Speranza, P., Valls, J.F., Guerra, G., Mazzella, C., 2005. Localization of the 5S and 45S rDNA sites and cpDNA sequence analysis in species of the Quadrifaria group of *Paspalum* (Poaceae, Paniceae). Ann. Bot. 96, 191–200.
Zuloaga, F.O., Morrone, O., 2005. Revisión de las especies de *Paspalum* para América del Sur austral (Argentina, Bolivia, sur del Brasil, Chile, Paraguay y Uruguay). Monogr. Syst. Bot. Mo. Bot. Gard. 102, 1–297.